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TITLE: Prediction of Breast Cancer Risk by Aberrant Methylation
in Mammary Duct Lavage

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13. ABSTRACT (Maximum 200 Words) Women found to have atypical hyperplasia on a breast biopsy are at significantly increased risk for breast cancer. Nipple duct lavage (NDL) is being promoted by some as a new screening test for atypical hyperplasia, but cytological interpretation is a subjective art and experience indicates that the underlying conditions represented by cytological atypia on NDL can range from intraductal papilloma to ductal carcinoma in situ (DCIS). Laboratory studies indicate that methylation of tumor suppressor genes is an early event in breast carcinogenesis and often represents a cell's attempt to defeat cell cycle control. We have shown previously that methylation of RASSF1A of APC in benign breast epithelium correlates with calculated breast cancer risk and the finding of Cyclin D2 methylation is specific for malignant transformation. We are applying these objective methylation tests to cells obtained by NDL from women with breast cancer, women at increased risk of breast cancer, and women at low or average risk of breast cancer. Successful completion of the project will provide new tools for the objective evaluation of breast epithelial cells obtained by NDL in order to accurately risk stratify women and to enhance the early detection of DCIS.				
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INTRODUCTION

While the lifetime risk for developing breast cancer is relatively high in all women, a subset of women are at increased risk. Risk can be estimated by computer software using history, pathological findings, previous history of breast cancer and other factors. We have developed custom software for risk estimation of women attending our high risk clinic. However, refinement of risk estimation remains an important goal.

Obtaining tissue for pathological examination usually requires invasive procedures such as needle aspiration or surgical biopsy. A recently developed, minimally invasive technique, nipple duct lavage (NDL) extensively samples the ductal/lobular system (where all breast carcinomas arise) and provides adequate cell numbers for pathologic and laboratory studies.

Several intermediate markers have been utilized for risk assessment. We are testing for aberrant methylation of a panel of genes frequently silenced in breast cancers. In other cancers it has been demonstrated that aberrant methylation commences early during multistage carcinogenesis, even in histologically normal epithelium. Thus it is likely that it will be an early event in breast cancer pathogenesis. Utilizing a panel of markers will permit us to utilize data from individual markers as well as a methylation index based on the combined results.

We are performing bilateral NDL on three groups of women (50 in each group) over the course of three years. The groups will consist of a) women with cancer; and women with computer estimated b) high risk and c) low risk. Because data from the contralateral breasts of cancer patients will be analyzed separately, our findings will represent materials from breasts at four levels of risk (beyond risk, very high risk, high risk and low/average risk).

Thus, we will be able to compare and contrast three methods of risk assessment (computer generated modelling/breast cancer diagnosis, cytological examination and aberrant methylation) in women at four levels of risk. We will determine whether cytologic examination of NDL fluids alters computer generated risk assessment. The finding of aberrant methylation in women at low risk without cytologic changes, may help identify a subgroup at increased risk not identified by the other techniques.

Our results will help assess the newly developed technique of NDL, both for cytological diagnosis and utilization of intermediate markers. The data may provide important new information that helps refine breast cancer assessment. Women identified at increased risk will benefit from increased surveillance and chemoprevention. Thus, our proposal is of direct relevance to the clinical management of women at increased risk, and may, ultimately, help reduce the incidence of invasive cancer.

BODY

Recruitment and Sampling

Recruitment of study subjects and performance of nipple duct lavage sampling is slightly behind schedule for meeting our accrual goals in the time specified. This is due to two administrative hurdles which have severely impacted the conduct of the study this year. The first was a very long delay in having our first annual report approved which delayed receipt of the second year of funding. Because accrual was brisk the first year we exhausted our supply budget and had to suspend accrual while waiting for the second

year of funding. Soon after resuming accrual the second year, the principal investigator, Arvind Virmani, decided to leave the institution. Despite submitting all of the necessary paperwork to designate a new principal investigator in January 2003, the DoD has still not acted on the request. Nevertheless, to date we have accrued and sampled 75 of the 150 subjects specified by the protocol. Comprehensive risk factor information has been electronically archived on all of the patients as has cytological results for nearly all of the patients. We are deferring methylation analysis until the third year of the study so that we can batch analyze all of the samples using the same reagents, markers and methods. We would anticipate completing, or nearly completing accrual and sampling in this, the third and final year of the study. There have been no adverse events.

Finalization of the Panel of Genes to Evaluate

One important objective of the study was to assemble a panel of genes whose methylation status could contribute to risk stratification or early diagnosis of breast cancer. To achieve this, we have been screening archival benign and malignant breast epithelial cells using a panel of six genes. We have determined from this that methylation of RASSF1A or APC correlates with breast cancer risk calculated using the validated Gail model and that methylation of Cyclin D2 occurs in 50% of breast cancers and is a cancer-specific marker. We are continuing to add genes to the panel with a focus on methylation of genes that code for secreted chemokines, such as HIN-1, which have recently emerged as important differentiation markers for benign breast cells.

Optimization of Laboratory Methods

Though nipple duct lavage often retrieves millions of epithelial cells it sometimes returns only dozens of cells. Though paucicellular samples render cytological assessment almost useless, there is no reason why these samples should limit a molecular analysis. We systematically optimized our DNA extraction protocols using real time PCR for housekeeping genes as a measure of amplifiable DNA yield. After comparing four protocols we have settled on a Dynabead system for ensuring extraction of ample quantities of high quality DNA from even our most paucicellular NDL samples.

Though we have already optimized a methylation specific PCR for this study, there have been several quantitative methylation assays recently described. We are currently evaluating these methods with the intention of optimizing a high throughput quantitative assay for this study that can accommodate up to 10 markers per multiplex reaction.

Interim Analysis and Findings

Interim analysis has focused on quality of the samples and cytological findings. A recent review of the first 51 patients, which included 24 women with breast cancer showed that, of the 97 breast we lavaged, 82 (74%) returned enough epithelial cells for a cytological diagnosis. Atypia was identified in 28% of these 82 breasts which is generally consistent with atypia rates published by other investigators. Of note, atypia was not more common in lavage samples from breasts with an untreated cancer than from breasts with no cancer (29% vs 27%). Atypia did not correlate with age, phase of the menstrual cycle at sampling, presence of expressible fluid discharge in the duct sampled, calculated breast cancer risk or the use of hormone medications. Of note, 90% of the

cases diagnosed as atypical were from premenopausal subjects who accounted for only 57% of the total sample ($P = 0.38$). A few high risk patients were lavaged at the time of prophylactic mastectomy and the histological correlates of lavage atypia in mammographically normal breasts have ranged from a single small focus of atypical lobular hyperplasia to extensive ductal carcinoma in situ. These findings make it clear that cytology is too subjective and lacks sufficient specificity for optimal assessment of epithelial cells obtained by nipple duct lavage. It is anticipated that molecular analysis will significantly enhance the characterization of these samples.

KEY RESEARCH ACCOMPLISHMENTS

- Promoter region methylation of RASSF1A and APC in benign breast epithelium correlates with breast cancer risk calculated using a validated mathematical model and may provide an objective measure for risk stratifying duct lavage samples.
- Promoter region methylation of Cyclin D2 is a cancer-specific change that, if applied to duct lavage samples, may contribute to the diagnosis of mammographically occult DCIS.
- Atypia is diagnosed at a similar rate whether fluid producing or dry ducts are lavaged.
- Cytological assessment of duct lavage samples is unable to distinguish between breasts with cancer and breasts without cancer.

REPORTABLE OUTCOMES

Abstract and presentation Society of Surgical Oncology, 2003¹

Abstract and presentation Association for Academic Surgery, 2003²

CONCLUSIONS

Preliminary analysis of data from the first two years of this study highlights the limitations of cytological assessment as the sole modality for evaluation of nipple duct lavage samples. Preparatory work with methylation markers strongly suggests that the addition of molecular tests to the evaluation of nipple duct lavage samples will provide an objective approach for risk stratification and for the early detection of mammographically occult DCIS.

REFERENCES

¹ Euhus DM, Ashfaq R, Milchgrub S, Naftalis E, Leitch AM, Virmani A. Comparison of Nipple Duct Lavage & Random Fine Needle Aspiration Biopsy for the Detection of Atypical Breast Epithelium. Soc Surg Oncol, Los Angeles, CA, March 2003.

² Cler LR, Ashfaq R, Naftalis E, Leitch AM, Hoover S, Euhus DM, Cytological Atypia Diagnosed by Nipple Duct Lavage: Reproducibility and MRI Findings, Assc Acad Surg, Sacramento, CA 2003.

Comparison of Nipple Duct Lavage and Random Fine Needle Aspiration Biopsy for the Detection of Atypical Breast Epithelium

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Women with atypical breast cells identified by nipple duct aspiration or by random fine needle aspiration biopsy (FNA) are at increased risk for breast cancer. Nipple duct lavage (NDL) has been proposed as a screening method for the detection of atypical breast epithelium, but the sensitivity of the procedure for the detection of atypia is unknown. We performed both nipple duct lavage and random one-site FNA bilaterally in 21 breast cancer patients and 27 asymptomatic women (12 average risk and 15 increased risk) and compared insufficient sample rates and atypia prevalence for the two procedures. There were 71 evaluable breasts (including 21 unaffected breasts contralateral to a breast cancer). The insufficient sample rate was 14/71 (19.7%) for NDL compared with 10/71 (14.1%) for FNA ($P = 0.26$). Mild or marked atypia was identified in 18/71 (25%) breasts by NDL compared with 4/71 (6%) by FNA ($P = 0.002$). Of the four cases with atypia detected by FNA, one was insufficient by NDL, one showed scant normal epithelial cells and two showed moderate or abundant cells (100 to 999 and >1000 cells respectively) consistent with epithelial hyperplasia. Though insufficient sample rates are similar for NDL and FNA, NDL is superior for the detection of atypical breast epithelium. Because one-site FNA increased the atypia rate for the series from 18/71 (25%) to 22/71 (31%) the maximum sensitivity of NDL can be estimated at 82%. A more thorough approach to random FNA (e.g. four quadrant periareolar sampling) may have identified additional breasts with atypia that had been missed by NDL.

Cler LR, Ashfaq R, Naftalis E, Leitch AM, Hoover S, Euhus DM, Cytological Atypia Diagnosed by Nipple Duct Lavage: Reproducibility and MRI Findings

Body: Atypical cells in a nipple duct lavage (NDL) sample are thought to indicate an increased risk for breast cancer similar to atypical duct hyperplasia diagnosed by tissue biopsy. In reality the intraductal lesions that can give rise to an atypical lavage range from benign proliferative conditions, such as intraductal papilloma, to DCIS.

We performed repeat lavage and breast MRI scans on patients with atypical cells detected by NDL but with normal mammograms. 118 breasts from 62 women underwent NDL, and atypical cells were identified in 30 (25%) breasts from 22 (35%) of these women.

The atypia was mild in 19 (63%) breasts and marked in 11 (37%) breasts. Seven of the 30 (23%) breasts with atypical NDL underwent repeat lavage at an interval of 3 - 12 months, and atypia was confirmed in only three breasts (43%). Nineteen of the 30 (63%) breasts with an initial atypical lavage underwent breast MRI scans. Of these, 28 were interpreted as normal, 1 showed a region of borderline abnormal enhancement, and 1 was suspicious for malignancy. Both cases with abnormal MRI had markedly atypical cytology that was confirmed on repeat NDL. A total mastectomy specimen from the patient with the markedly atypical NDL and the suspicious MRI revealed 10 cm of high grade DCIS.

These data suggest that, in our hands, the finding of atypia with NDL is reproducible in fewer than half of the cases in which it is diagnosed, that MRI is unlikely to be abnormal in cases with mild atypia only, and that marked atypia may represent DCIS. Repeat lavage after a diagnosis of atypia may be indicated. However, breast MRI is recommended if the lavage is interpreted as markedly atypical.